PCT/GB 00/03660.

10/088801

GB00/3660

INVESTOR IN PEOPLE

The Patent Office
Concept House
Cardiff Road
Newport
South Wales
NP10 8QQ

4

I, the undersigned, being an officer duly authorised in accordance with Section 74(1) and (4) of the Deregulation & Contracting Out Act 1994, to sign and issue certificates on behalf of the Comptroller-General, hereby certify that annexed hereto is a true copy of the documents as originally filed in connection with the patent application identified therein.

In accordance with the Patents (Companies Re-registration) Rules 1982, if a company named in this certificate and any accompanying documents has re-registered under the Companies Act 1980 with the same name as that with which it was registered immediately before re-registration save for the substitution as, or inclusion as, the last part of the name of the words "public limited company" or their equivalents in Welsh, references to the name of the company in this certificate and any accompanying documents shall be treated as references to the name with which it is so re-registered.

In accordance with the rules, the words "public limited company" may be replaced by p.l.c., plc, P.L.C. or PLC.

Re-registration under the Companies Act does not constitute a new legal entity but merely subjects the company to certain additional company law rules.

Signed P. Mahoney

Dated 6 October 2000

PRIORITY DOCUMENT

SUBMITTED OR TRANSMITTED IN COMPLIANCE WITH RULE 17.1(a) OR (b)

Patents Form 1/77

Patents Act ..77
(Rule 16)

The Patent Office

1/77

Request for grant of a patent

(See the notes on the back of this form. You can also get an explanatory leaflet from the Patent Office to help you fill in this form)

24 SEP 1999

The Patent Office

Cardiff Road
Newport
Gwent NP9 1RH

1.	Your reference	KENF / P21403GB
2.	Patent application number *(The Patent Office will fill in this part)*	**9922505.4**
3.	Full name, address and postcode of the or of each applicant *(underline all surnames)*	The Mathilda and Terence Kennedy Institute of Rheumatology 1 Aspenlea Road Hammersmith London W6 8LH United Kingdom
	Patents ADP number *(if you know it)*	07416621001
	If the applicant is a corporate body, give the country/state of its incorporation	United Kingdom
4.	Title of the invention	THERAPEUTIC METHODS AND COMPOUNDS
5.	Name of your agent *(if you have one)* "Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*	**ERIC POTTER CLARKSON** **PARK VIEW HOUSE** **58 THE ROPEWALK** **NOTTINGHAM** **NG1 5DD**
	Patents ADP number *(if you know it)*	**1305010**

6.	If you are declaring priority from one or more earlier patent applications, give the country and the date of filing of the or of each of these earlier applications and *(if you know it)* the or each application number	Country	Priority application number *(if you know it)*	Date of filing (day / month / year)
7.	If this application is divided or otherwise derived from an earlier UK application, give the number and the filing date of the earlier application	Number of earlier application		Date of filing (day / month / year)

8. Is a statement of inventorship and of right to grant of a patent required in support of this request? *(Answer 'Yes' if:* YES

a) *any applicant named in part 3 is not an inventor; or*
b) *there is an inventor who is not named as an applicant, or*
c) *any named applicant is a corporate body. See note (d))*

9. Enter the number of sheets for any of the following items you are filing with this form. Do not count copies of the same document

Continuation sheets of this form	
Description	45
Claims(s)	5
Abstract	1
Drawing(s)	7

10. If you are also filing in any of the following, state how many against each item.

Priority Documents	0
Translations of priority documents	0
Statement of inventorship and right to grant of a patent *(Patents Form 7/77)*	NO
Request for preliminary examination and search *(Patents Form 9/77)*	NO
Request for substantive examination *(Patents Form 10/77)*	NO
Any other documents *(please specify)*	

11. I/We request the grant of a patent on the basis of this application.

Eric Potter Clarkson

Signature: **ERIC POTTER CLARKSON** Date: 23 September 1999

12. Name and daytime telephone number of person to contact in the United Kingdom: **0115 9552211**

Warning

After an application for a patent has been filed, the Comptroller of the Patent Office will consider whether publication or communication of the invention should be prohibited or restricted under Section 22 of the Patents Act 1977. You will be informed if it is necessary to prohibit or restrict your invention in this way. Furthermore, if you live in the United Kingdom, Section 23 of the Patents Act 1977 stops you from applying for a patent abroad without first getting written permission from the Patent Office unless an application has been filed at least 6 weeks beforehand in the United Kingdom for a patent for the same invention and either no direction prohibiting publication or communication has been given, or any such direction has been revoked.

Notes

a) *If you need help to fill in this form or you have any questions, please contact the Patent Office on 01645 500505.*
b) *Write your answers in capital letters using black ink or you may type them.*
c) *If there is not enough space for all the relevant details on any part of this form, please continue on a separate sheet of paper and write "see continuation sheet" in the relevant part(s). Any continuation sheet should be attached to this form.*
d) *If you have answered 'Yes' Patents Form 7/77 will need to be filed.*
e) *Once you have filled in the form you must remember to sign and date it.*
f) *For details of the fee and ways to pay please contact the Patent Office.*

THERAPEUTIC METHODS AND COMPOUNDS

Field of the invention

The present invention relates to methods of treatment of chronic inflammatory diseases and compounds for use in the same. In particular, the present invention relates to methods of identifying a compound with efficacy in the treatment of a chronic inflammatory disease.

Background to the invention

Chronic inflammatory diseases such as rheumatoid arthritis, Crohn's disease, multiple sclerosis and atherosclerosis are diseases in which the body's immune system produces anti-self (*i.e.* autoimmune) responses. In the case of rheumatoid arthritis, the clinical syndrome is characterised by relapsing/remitting inflammation within the synovial membrane, associated with progressive, erosive destruction of adjacent cartilage and bone. On a cellular level, these events are coincident with chronic infiltration of the synovial membrane with T cells, plasma cells and macrophages.

There is mounting evidence to implicate T cells in the initiation and perpetuation of chronic inflammatory diseases such as rheumatoid arthritis (RA). For example, patients with RA have been found to carry a specific polymorphism in the class II major histocompatibility complex (MHC), which is believed to render them genetically predisposed to developing RA (Panayi *et al.*, 1992, *Arthritis Rheum.* **35**:729-735). A key function

of class II MHC is to present antigen to a subpopulation of T cells, termed T helper cells, which are characterised by CD4+ surface markers. It is proposed that antigen presentation results in clonal expansion and activation of this population of T cells, which in turn leads to stimulation of other populations of synovial cells, such as macrophages, and the consequent release of pro-inflammatory cytokines. To date, however, the nature of the environmental stimulus that triggers RA remains unknown.

In addition to a role in initiation of RA, T helper cells are also proposed to be important in the perpetuation of this disease, either by becoming activated against self-proteins (*e.g.* peptides derived from the degradation of MHC molecules and/or structural proteins in the joint) or by being re-exposed to the initiating antigen. This further activation of T cells is suggested to result in the sustained release of pro-inflammatory cytokines, as well as enzymes that mediate destruction of the cartilage and bone.

As a consequence of the growing evidence of an involvement of T cells in the pathogenesis of RA, several treatments of this disease have been developed which target T cell via their surface markers, for example anti-CD4 antibody therapy, anti-CD52 antibody therapy, anti-CD5 antibody therapy and anti-interleukin-2 receptor antibody therapy (*e.g.* van der Lubbe *et al.*, 1995, *Arthritis Rheum.* **38**:1097-1106; Weinblatt *et al.*, 1995, *Arthritis Rheum.* **38**:1589-1594; Olsen *et al.*, 1996, *Arthritis Rheum.* **39**:1102-1108; Moreland *et al.*, 1995, *Arthritis Rheum.* **38**:1177-1186). However, the results of clinical trials with such therapies have been largely disappointing, as a result of a lack of efficacy and/or the presentation of toxicity-related side effects. These limitations are thought

to be due, at least in part, to the lack of specificity of the therapies for the particular population of T cells responsible for inducing production of pro-inflammatory cytokines by macrophages in RA joints.

An alternative approach for developing new treatments of RA has been to target the pro-inflammatory cytokines produced in the synovium by macrophages in response to T cell activation. Although several pro-inflammatory cytokines may be released following T cell activation in RA, tumour necrosis factor α (TNFα) has attracted particular attention. The importance of TNFα was initially demonstrated using dissociated rheumatoid joint cell cultures (Buchan *et al.*, 1988, *Clin. Exp. Immunol.* **73**:449-455;) and subsequently confirmed in animal models of arthritis (Thorbecke *et al.*, 1992, *Proc. Natl. Sci. USA* **89**:7375-7379; Williams *et al.*, 1992, *Proc. Natl. Sci. USA* **89**:9784-9788). The findings of these pre-clinical studies led to successful clinical trials of anti-TNFα antibody therapy, establishing the importance of TNFα as a therapeutic target (*e.g.* Elliott *et al.*, 1993, *Arthritis Rheum.* **36**:1681-1690; Elliott *et al.*, 1994, *Lancet* **344**:1105-1110; Elliott *et al.*, 1994, *Lancet* **344**:1125-1127).

In RA joints, the cells responsible for the majority of TNFα production are macrophages (Chu *et al.*, 1991, *Arthritis Rheum.* **34**:1125-1132). Hence, there is much interest in understanding the mechanisms underlying the regulation of TNFα production in cells of this lineage. Available data suggest that both water-soluble factors and cell-cell interactions may be involved in mediating T cell-induced production of TNFα by macrophages/monocytes.

Sebbag *et al.* (1997) *Eur. J. Immunol.* **27**:624-632 recently demonstrated that cytokine stimulation (using IL-15 alone, or a cocktail of IL-6, TNFα and IL-2) could activate a specific subset of T helper cells termed cytokine stimulated T cells ('T_{cy} cells'), which in turn could induce TNFα production (but not IL-10 production) in monocytes. Stimulation of conventional T cell receptor-stimulated T cells ('T_{tcr} cells') using anti-CD3 antibodies also induced TNFα production by monocytes, in addition to the production of an anti-inflammatory cytokine, IL-10 (Parry *et al.*, 1997, *J. Immunol.* **158**:3673-3681). In the case of both T_{cy} and T_{tcr} cell stimulation, TNFα production was found to be dependent on cell-cell interactions between T cells and macrophages. On the basis of these findings, Sebbag *et al.* postulated that T_{cy} cells might contribute to the production of pro-inflammatory cytokines in RA synovial tissue, thus contributing to the relative imbalance of pro-inflammatory cytokines (*e.g.* TNFα) over anti-inflammatory cytokines (*e.g.* IL-10) in such tissue.

Interleukin-15 (IL-15) has also been implicated in the production of TNFα in RA. This pro-inflammatory cytokine, known to be present in RA synovium (McInnes *et al.*, 1996, *Nature Medicine* **2**:175-182), has been shown to activate peripheral blood T cells which, in turn, are able to induce TNFα production in U937 cells and adherent RA synovial cells in a contact-dependent manner (McInnes *et al.*, 1997, *Nature Medicine* **3(2)**:189-195).

To date, however, the identity of the T cell population(s) that mediate(s) the production of pro-inflammatory cytokines in RA synovial tissue

remains unclear. Furthermore, a means of selectively targeting such cells is absent.

Hence, the present invention seeks to provide a method of identifying compounds with efficacy in the treatment of a chronic inflammatory disease, for example compounds which selectively target the population of T cells that induce production of pro-inflammatory cytokines by macrophages/monocytes in RA synovial tissue.

Summary of the invention

A first aspect of the present invention is a method of treatment of a chronic inflammatory disease in a patient, the method comprising the administration to the patient of a compound that selectively inhibits T_{cy} cells.

In a preferred embodiment, said compound selectively inhibits T_{cy} cell-induced release of one or more pro-inflammatory cytokines from monocytes. Preferably, the cytokine is tumour necrosis factor-α.

Conveniently, said compound selectively inhibits NF-κB.

Advantageously, said compound selectively activates PI3 kinase.

Another aspect of the present invention provides a method of identifying a compound with efficacy in the treatment of a chronic inflammatory

disease comprising the step of testing said compound for an ability to selectively inhibit T_{cy} cells.

It will be appreciated by persons skilled in the art that such a compound may be a drug-like compound or lead compound for the development of a drug-like compound.

The term "drug-like compound" is well known to those skilled in the art, and includes a compound having characteristics that may make it suitable for use in medicine, for example as the active ingredient in a medicament. Thus, for example, a drug-like compound may be a molecule that may be synthesised by the techniques of organic chemistry, molecular biology or biochemistry, and is preferably a small molecule, which may be of less than 5000 daltons and which may be water-soluble. A drug-like compound may additionally exhibit features of selective interaction with a particular protein or proteins and be bioavailable and/or able to penetrate target cellular membranes, but it will be appreciated that these features are not essential.

The term "lead compound" is similarly well known to those skilled in the art, and may include a compound which, whilst not itself suitable for use as a drug (for example because it is only weakly potent against its intended target, non-selective in its action, unstable, poorly soluble, difficult to synthesise or has poor bioavailability), may provide a starting-point for the design of other compounds that may have more desirable characteristics.

By "efficacy in the treatment of a chronic inflammatory disease" we include efficacy in the therapeutic and/or prophylactic treatment of a chronic inflammatory disease, for example an ability to prevent the onset and/or progression of said disease, and/or alleviate the symptoms of said disease.

By "an ability to selectively inhibit T_{cy} cells" we include an ability to inhibit, either directly or indirectly, the function of T_{cy} cells to a greater extent than other T cell populations, such as T_{tcr} cells. The inhibition may leave the T_{cy} population intact (but functionally inhibited) or it may reduce the number of T_{cy} cells present, for example by selectively killing them.

A functional inhibition of T_{cy} cells includes inhibiting the interaction of T_{cy} cells with monocytes/macrophages, for example the direct cell-cell interaction between T_{cy} cells and monocytes/ macrophages that induces the production of pro-inflammatory cytokines by monocytes/macrophages.

A further aspect of the present invention provides a method of identifying a compound with efficacy in the treatment of a chronic inflammatory disease comprising the step of testing said compound for an ability to selectively inhibit T_{cy} cell-induced release of one or more pro-inflammatory cytokines from monocytes/macrophages.

Advantageously, the one or more pro-inflammatory cytokines is or includes tumour necrosis factor α (TNFα). Conveniently, the one or more pro-inflammatory cytokines is or includes IL-15.

By "a chronic inflammatory disease" we include any disease in which there is a progressive and sustained anti-self (*i.e.* autoimmune) response, typically leading to the development of tissue inflammation and, in severe cases, destruction of said tissue.

Preferably, the chronic inflammatory disease is a disease of humans. Such diseases include, but are not limited to, rheumatoid arthritis, Crohn's disease, multiple sclerosis and atherosclerosis. In principle, the invention is applicable to other mammals and birds, including pets such as dogs and cats and agriculturally important animals such as cows, horses, sheep, pigs, chickens and turkeys.

Advantageously, the chronic inflammatory disease is rheumatoid arthritis.

It will be understood that it is desirable to identify compounds that may modulate the function of T_{cy} cells *in vivo*. Thus, it will be understood that reagents and conditions used in the methods of the invention may be chosen such that the interactions between said compounds and T_{cy} cells are substantially the same as would occur *in vivo*.

In principle, one may use any test for inhibition of T cells, and one determines whether the inhibition is greater in the case of T_{cy} cells than other T cells, especially T_{tcr} cells.

In a preferred embodiment of the methods of identifying a compound of the present invention, testing the compound for an ability to selectively inhibit T_{cy} cells or selectively inhibit T_{cy} cell-induced release of one or

more pro-inflammatory cytokines from monocytes comprises the following steps:

(i) pre-incubating separate cultures of T_{cy} cells and T_{tcr} cells with a compound to be tested;

(ii) resuspending said T_{cy} cells and T_{tcr} cells in the absence of the test compound;

(iii) stimulating monocytes by co-culturing with said resuspended T_{cy} cells and T_{tcr} cells; and

(iv) assaying for TNFα production by said stimulated monocytes.

Selective compounds of the present invention include those compounds which selectively inhibit TNFα production by monocytes/macrophages stimulated with T_{cy} cells to a greater extent than they inhibit TNFα production by monocytes/macrophages stimulated with T_{tcr} cells. Preferably, T_{cy} cell-stimulated TNFα production is reduced to no more than 50% compared to TNFα production by monocytes stimulated with T_{cy} cells which are not pre-incubated with the test compound, preferably no more than 20%, 10%, 5% or 1%. Ideally, T_{cy} cell-stimulated TNFα production is substantially zero and TNFα production by monocytes/ macrophages stimulated with T_{tcr} cells is substantially unaffected.

In an further preferred embodiment of the methods of identifying a compound of the present invention, testing the compound for an ability to selectively inhibit T_{cy} cells or selectively inhibit T_{cy} cell-induced release of one or more pro-inflammatory cytokines from monocytes comprises assaying for NF-κB inhibition in monocytes.

By "NF-κB inhibition" we include one or more of the following:

(i) a reduction in expression of NF-κB-dependent genes;
(ii) a reduction in the ability for NF-κB to bind to its promoter on DNA;
(iii) a decrease in translation of NF-κB; and/or
(iv) a decrease in transcription of NF-κB.

Conveniently, NF-κB inhibition is deemed to exist if, when monocytes are incubated in culture with a compound, and nuclear extracts are prepared from the monocytes, the nuclear extracts have a reduced binding to the NFκB promoter on DNA oligonucleotides. The latter can be shown using an electrophoretic mobility shift assay (EMSA). Preferably, the binding is reduced to no more than 50% compared to the binding of nuclear extracts prepared from monocytes that have not been pre-incubated with the test compound, preferably no more than 20%, 10%, 5% or 1%. Ideally, the binding is substantially zero.

An exemplary EMSA is described in Clarke *et al.* (1995) *Eur. J. Immunol.* **25**:2961-2966.

Advantageously, NF-κB inhibition is deemed to exist if, in a reporter gene assay wherein the NF-κB gene (or at least the promoter thereof) is coupled to a β-galactosidase gene in a cell line, β-galactosidase activity in the cell lysates is reduced following incubation of the cells with a test compound. Preferably, β-galactosidase activity is reduced to no more than 50% compared to β-galactosidase activity in lysates from cells which

have not been incubated with the test compound, and more preferably to no more than 20%, 10%, 5% or 1%. Ideally, β-galactosidase activity is substantially zero.

An exemplary reporter gene assay is described in Clarke *et al.* (1995) *Eur. J. Immunol.* **25**:2961-2966.

In an alternative preferred embodiment of the methods of identifying a compound of the present invention, testing the compound for an ability to selectively inhibit T_{cy} cells or selectively inhibit T_{cy} cell-induced release of one or more pro-inflammatory cytokines from monocytes comprises assaying for PI3 kinase activation in monocytes.

Conveniently, PI3 kinase activation is deemed to exist if, when monocytes are incubated in culture with a compound, PI3 kinase activity is increased. This may be determined by lysing the monocytes, harvesting the supernatant, immunoprecipitating PI3 kinase from the supernatant, and assaying the immunoprecipitate for PI3 kinase activity.

The monocytes in such case may be human peripheral blood monocytes or a monocyte cell line, such as Mono Mac-6 (Zeigler-Heitbrock *et al*, *Int J Cancer* 1988, 41, 456-461).

It will be appreciated that screening assays which are capable of high throughput operation will be particularly preferred. Examples may include cell-based assays and protein-protein binding assays. An SPA-based (Scintillation Proximity Assay; Amersham International) system

may be used. For example, an assay for identifying a compound capable of modulating the activity of a protein kinase may be performed as follows. Beads comprising scintillant and a polypeptide that may be phosphorylated may be prepared. The beads may be mixed with a sample comprising the protein kinase and ^{32}P-ATP or ^{33}P-ATP, together with the test compound. Conveniently this is done in a 96-well format. The plate is then counted using a suitable scintillation counter, using known parameters for ^{32}P or ^{33}P SPA assays. Only ^{32}P or ^{33}P that is in proximity to the scintillant, *i.e.* only that bound to the polypeptide, is detected. Variants of such an assay, for example in which the polypeptide is immobilised on the scintillant beads via binding to an antibody, may also be used.

A further method of identifying a compound that is capable of binding to the polypeptide (such as NF-κB or PI3 kinase) is one where the polypeptide is exposed to the compound and any binding of the compound to the said polypeptide is detected and/or measured. The binding constant for the binding of the compound to the polypeptide may be determined. Suitable methods for detecting and/or measuring (quantifying) the binding of a compound to a polypeptide are well known to those skilled in the art and may be performed, for example, using a method capable of high throughput operation, for example a chip-based method. New technology, called VLSIPS™, has enabled the production of extremely small chips that contain hundreds of thousands or more of different molecular probes. These biological chips or arrays have probes arranged in arrays, each probe assigned a specific location. Biological chips have been produced in which each location has a scale of, for example, 10 μm. The chips can be

used to determine whether target molecules interact with any of the probes on the chip. After exposing the array to target molecules under selected test conditions, scanning devices can examine each location in the array and determine whether a target molecule has interacted with the probe at that location.

Biological chips or arrays are useful in a variety of screening techniques for obtaining information about either the probes or the target molecules. For example, a library of peptides can be used as probes to screen for drugs. The peptides can be exposed to a receptor, and those probes that bind to the receptor can be identified. See US Patent No. 5,874,219 issued 23 February 1999 to Rava *et al.*

According to a further aspect of the present invention, there is provided a compound identifiable or identified by the methods of the first or second aspect of the invention, for use in medicine. Such compounds include tyloxapol (Ghio *et al*, *Am J Respir Crit Care Med* 1996, **154**, 783-8), panepoxydone (Erkel *et al*, *Biochem Biophys Res Commun* 1996, **226**, 214-21), emodin (Kumar *et al*, *Oncogene* 1998, 17, 913-9138), anetholdithiolthione (Sen *et al*, *Biochem Biophys Res Commun* 1996, **218**, 148-53), retinoids (Na *et al*, *J Biol Chem* 1999, **274**, 7674-80), phenylalanine chloromethyl ketone (PCK) (Jeong *et al*, *Immunology* 1997, **92**, 267-73), sanguinarine (Chaturvedi *et al*, *J Biol Chem* 1997, **272**, 30129-34), Δ^9-tetrahydrocannabinol (Jeon *et al*, *Mol Pharmacol* 1996, **50**, 334-341), gliotoxin, sesquiterpene lactones (Hehnert *et al, J Biol Chem* 1998, **272**, 1288-97; Lyss *et al*, *J Biol Chem* 1998, **273**, 33508-16), caffeic acid phenethyl ester (Natarajau *et al*, *Proc. Nat. Acad. Sci. USA,*

1996, **93**, 9090-5), pyrrolidine dithiocarbamate (Schreck *et al*, *J Exp Med* 1992; **175**: 1181-94), lovastatin (Merck), azelastine HC1 (Azeptin, Eisai Co. Tokyo), tepoxalin (Kazmi *et al*, *J Cell Biochem* 1995; **57**: 299-310), epigallocatechin-3-gallate (Lin & Lin, *Mol Pharmacol* 1997; **52**: 465-72), deoxyspergualin (Bristol-Myers Squibb), phenyl-*N*-*tert*-butylnitrone (Aldrich Chemical Co), quercetin (Sato *et al*, *J Rheumatol* 1997; **24**: 1680-4), curcumin, E3330 (a quinone derivative Tsukuba Research Labs, Eisai Co. Japan), and proteasome inhibitors, such as PSI (Calbiochem), ALLN (Boehringer Mannheim), lactacystin (Delic *et al*, *Br. J. Cancer* 1998; **77**: 1130-7), MG-132 (Peptide International), X-LFF and the Calpain inhibitors reviewed by Beauparlant & Hiscott, *Cytokine & Growth Factor Reviews* 1996; **7**: 175-90, and CVT-634 (CV Therapeutics, Palo Alto).

It will be appreciated that any compound according to the third aspect of the invention should be sufficiently non-toxic to allow use of the compound at a therapeutic dose.

In a preferred embodiment of the above aspect of the present invention, the compound selectively inhibits T_{cy} cell-induced release of one or more pro-inflammatory cytokines from monocytes. Conveniently, release of said cytokines is assayed by enzyme-linked immunosorbent assay (ELISA), for example as described in Sebbag *et al.* (1997) *Eur. J. Immunol.* **27**:624-632.

Advantageously, the one or more pro-inflammatory cytokine is or includes TNFα.

It will be appreciated that the compounds of the invention may decrease the activity of NF-κB, *e.g.* by binding substantially reversibly or substantially irreversibly to the active site of the NF-κB polypeptide. In a further example, the compound may bind to a portion of said polypeptide that is not the active site so as to interfere with the binding of the said polypeptide to its substrate. In a still further example, the compound may bind to a portion of said polypeptide so as to decrease said polypeptide's activity by an allosteric effect. This allosteric effect may be an allosteric effect that is involved in the natural regulation of the said polypeptide's activity, for example in the activation of said polypeptide by an "upstream activator".

It will also be understood that the compounds of the invention may decrease the activity of NF-κB by binding to any of the subunits of NF-κB, namely p65(RelA), RelB, cREL, p50, p52, p105 (precursor to p50) and p100 (precursor to p52).

In a further embodiment, the compound increases the activity of PI3 kinase, *e.g.* by binding substantially reversibly or substantially irreversibly to the active site of the of PI3 kinase polypeptide. Alternatively, the compound may bind to a portion of this polypeptide that is not the active site so as to aid the binding of the said polypeptide to its substrate. In a still further example, the compound may bind to a portion of said polypeptide so as to increase said polypeptide's activity by an allosteric effect. This allosteric effect may be an allosteric effect that is involved in the natural regulation of the said polypeptide's activity for

example in the activation of the said polypeptide by an "upstream activator".

Another aspect of the present invention provides a compound according to the third or fourth aspect of the invention for use in medicine.

Exemplary uses of the compounds of the invention in medicine include the use of said compounds in the prophylactic and/or therapeutic treatment of chronic inflammatory diseases, such as rheumatoid arthritis, Crohn's disease, multiple sclerosis and atherosclerosis.

The compounds of the invention may be delivered systemically or locally. They may be administered orally, intramuscularly, intravenously, intranasally or via the lung. In particular, they may be administered directly into the synovium (*i.e.* intra-articularly).

A further aspect of the present invention provides the use of a compound according to the fifth aspect of the invention in the preparation of a medicament for the treatment of a chronic inflammatory disease such as rheumatoid arthritis.

Yet another aspect of the present invention provides a pharmaceutical formulation comprising a compound of the invention and a pharmaceutically acceptable carrier. It will be appreciated that such formulations may be administered to a patient with a chronic inflammatory disease either alone or in combination with other therapeutic agents.

The formulations may conveniently be presented in unit dosage form and may be prepared by any of the methods well known in the art of pharmacy. Such methods include the step of bringing into association the active ingredient (*i.e.* compound of the invention) with a carrier which constitutes one or more accessory ingredients. In general the formulations are prepared by uniformly and intimately bringing into association the active ingredient with liquid carriers or finely divided solid carriers or both, and then, if necessary, shaping the product.

Formulations in accordance with the present invention suitable for oral administration may be presented as discrete units such as capsules, cachets or tablets, each containing a predetermined amount of the active ingredient; as a powder or granules; as a solution or a suspension in an aqueous liquid or a non-aqueous liquid; or as an oil-in-water liquid emulsion or a water-in-oil liquid emulsion. The active ingredient may also be presented as a bolus, electuary or paste.

A tablet may be made by compression or moulding, optionally with one or more accessory ingredients. Compressed tablets may be prepared by compressing in a suitable machine the active ingredient in a free-flowing form such as a powder or granules, optionally mixed with a binder (*e.g.* povidone, gelatin, hydroxypropylmethyl cellulose), lubricant, inert diluent, preservative, disintegrant (*e.g.* sodium starch glycolate, cross-linked povidone, cross-linked sodium carboxymethyl cellulose), surface-active or dispersing agent. Moulded tablets may be made by moulding in a suitable machine a mixture of the powdered compound moistened with

an inert liquid diluent. The tablets may optionally be coated or scored and may be formulated so as to provide slow or controlled release of the active ingredient therein using, for example, hydroxypropyl-methylcellulose in varying proportions to provide desired release profile.

Formulations suitable for topical administration on the skin include creams, gels and ointments comprising the active ingredient and a pharmaceutically acceptable carrier.

Formulations suitable for parenteral administration include aqueous and non-aqueous sterile injection solutions which may contain anti-oxidants, buffers, bacteriostats and solutes which render the formulation isotonic with the blood of the intended recipient; and aqueous and non-aqueous sterile suspensions which may include suspending agents and thickening agents. The formulations may be presented in unit-dose or multi-dose containers, for example sealed ampoules and vials, and may be stored in a freeze-dried (lyophilised) condition requiring only the addition of the sterile liquid carrier, for example water for injections, immediately prior to use. Extemporaneous injection solutions and suspensions may be prepared from sterile powders, granules and tablets of the kind previously described.

Preferred unit dosage formulations are those containing a daily dose or unit, daily sub-dose or an appropriate fraction thereof, of an active ingredient.

It should be understood that, in addition to the compounds particularly mentioned above, the formulations of this invention may include other agents conventional in the art having regard to the type of formulation in question, for example those suitable for oral administration may include flavouring agents.

Gene therapy may be employed, as in the model adenovirus-based AdvIκBα system disclosed herein, to introduce polynucleotides encoding molecules which selectively inhibit T_{cy} cells, for example inhibitors of the NFκB pathway.

EXAMPLES

The present invention will now be described in more detail with reference to the following non-limiting figures and examples:

Figure 1 shows the effect of a porous membrane insert on monocyte TNFα production induced by T_{tcr} cells and T_{cy} cells.

Human peripheral blood T cells were isolated and cultured with either (A) anti-CD3 antibodies for 24 hours to selectively stimulate T_{tcr} cells or (B) a cocktail of cytokines (IL-2, IL-6 and TNFα) for 8 days to selectively stimulate T_{cy} cells. Following fixation, T-cells were incubated with monocytes (at a ratio of 5:1 T cells:monocytes) for 18 hours in the absence or presence of a porous membrane insert which physically separated the two cell populations in the tissue culture plate. In control experiments, separate cultures of monocytes and T cells were analysed.

In addition, lipopolysaccharide (LPS, 10 ng/ml) was used as a positive control. In all experiments, culture supernatants were assayed for TNFα content. Data are expressed as means ± SD, and are representative of three experiments performed using different donors.

Figure 2 shows the effect of over-expression of the NF-κB inhibitor, IκBα, on monocyte TNFα production induced by T_{tcr} cells and T_{cy} cells. Human peripheral blood T cells were isolated and cultured in the absence of a stimulatory challenge ('unstimulated') or in the presence of (A) anti-CD3 antibodies for 24 hours to selectively stimulate T_{tcr} cells or (B) a cocktail of cytokines (IL-2, IL-6 and TNFα) for 8 days to selectively stimulate T_{cy} cells, prior to fixation. Monocytes were cultured with M-CSF (100 ng/ml) for 2 days prior to infection with adenovirus containing either IκBα (AdvIκBα) or no insert (Adv0), at a multiplicity of infection (m.o.i.) of 80:1. Fixed, activated T cells and M-CSF-treated monocytes were then co-cultured for 18 hours at a ratio of 5:1 (T cells:monocytes). In control experiments, M-CSF-treated monocytes were cultured in the absence of T cells. In all experiments, culture supernatants were assayed for TNFα content. Data are expressed as means ± SD, and are representative of three experiments performed using different donors.

Figure 3 shows the effect of PI3 kinase inhibitors on T cell-induced production of TNFα by monocytes.
Human peripheral blood T cells were isolated and cultured with either (A and C) anti-CD3 antibodies for 24 hours to selectively stimulate T_{tcr} cells or (B and D) a cocktail of cytokines (IL-2, IL-6 and TNFα) for 8 days to selectively stimulate T_{cy} cells. Following fixation, T-cells were incubated

with monocytes (at a ratio of 5:1 T cells:monocytes) for 18 hours in the presence of increasing concentrations of Wortmannin or LY294002. In all experiments, culture supernatants were assayed for TNFα content. Data are expressed as means ± SD, and are representative of three experiments performed using different donors.

Figure 4 shows the induction of monocyte TNFα production by T cells derived from rheumatoid arthritis (RA) synovial tissue.

CD3+ enriched cells were isolated from RA synovial mononuclear cells by direct, positive selection on anti-CD3 antibody coated Dynabeads. Following fixation, RA T cells were incubated for 18 hours with normal monocytes at increasing ratios of T cells to monocytes, as indicated, and in the absence or presence of a porous membrane insert which physically separated the two cell populations in the tissue culture plate. In control experiments, separate cultures of monocytes and T cells were analysed. Culture supernatants were assayed for TNFα content. Data are expressed as means ± SD, and are representative of three experiments performed using different donors.

Figure 5 shows the effect of over-expression of the NF-κB inhibitor, IκBα, on monocyte TNFα production induced by rheumatoid arthritis T cells.

CD3+ enriched cells were isolated from RA synovial mononuclear cells by direct, positive selection on anti-CD3 antibody coated Dynabeads. Following fixation, RA T cells were incubated for 18 hours (at a ratio of 3:1 T cells:monocytes) with normal monocytes treated with M-CSF (100 ng/ml) for 2 days and then infected with adenovirus containing either

IκBα (AdvIκBα) or no insert (Adv0) (m.o.i. from 20:1 to 80:1). Culture supernatants were assayed for TNFα content. Data are expressed as means ± SD, and are representative of three experiments performed using different donors.

Figure 6 shows the effect of PI3 kinase inhibitors on monocyte TNFα production induced by rheumatoid arthritis T cells.

CD3+ enriched cells were isolated from RA synovial mononuclear cells by direct, positive selection on anti-CD3 antibody coated Dynabeads. RA cells (1×10^6 cells/ml) were cultured in the absence ('untreated') or presence of increasing concentrations of (A) Wortmannin or (B) LY294002. In all experiments, culture supernatants were assayed for TNFα content. Data are expressed as means ± SD, and are representative of eight experiments performed.

T cell and monocyte purification

Human peripheral blood T cells and monocytes were isolated from single donor plateletpheresis residues (purchased from the North London Blood Transfusion service, Colindale, GB). Mononuclear cells were isolated by Ficoll/Hypaque centrifugation (specific density 1.077 g/ml, Nycomed Pharma AS, Oslo, Norway), prior to cell separation in a Beckman JE6 elutriator. Elutriation was performed in culture medium containing 1% heat-inactivated foetal calf serum (FCS) using methods known in the art (see Sebbag *et al.*, 1997, Parry *et al.*, 1997). T cell and monocyte purity were assessed by flow cytometry (see Sebbag *et al.*, 1997).

T cell fractions contained typically about 70% CD3-expressing cells, about 8% CD19-expressing cells, and less than 1% CD14-expressing cells. Monocyte fractions contained typically about 85% CD14-expressing cells, less than 0.5% CD19-expressing cells, and less than 3% CD3-expressing cells.

Isolation of rheumatoid arthritis synovial mononuclear cells and enrichment of CD3+ cells

Rheumatoid arthritis (RA) mononuclear cells were obtained from synovial tissue specimens provided by the Rheumatology Clinic, Charing Cross Hospital, London, UK. Tissue was teased into small pieces and digested in medium containing 0.15 mg/ml DNase type 1 (Sigma, UK) and 5 mg/ml collagenase (Roche, UK) for 1 to 2 hours at 37°C. After passing cells through nylon mesh to exclude cell debris, cells were washed and resuspended in RPMI medium (BioWhittaker, Verviers, Belgium) (containing 10% heat-inactivated FCS) at a density of 1 x 10^6 cells/ml and used immediately. CD3+ enriched cells were isolated from RA synovial mononuclear cells by direct, positive selection using Dynabeads (Dynal UK Ltd, Wirral, UK) coated with anti-CD3 monoclonal antibodies (anti-CD3 mAbs). Briefly, mononuclear cells (1 x 10^7) were cultured with 100 μl anti-CD3 mAb-coated Dynabeads for 20 minutes at 4°C under constant rotation. Cells attached to beads were isolated using a magnetic particle concentrator (Dynal, Merseyside, UK) and cultured for 6 hours at 37°C. Detached cells were removed from magnetic beads and washed three times using the magnetic particle concentrator.

This technique allowed for isolation of CD3+ cells, yielding cells of high purity (>99%) and viability (>95%). Following isolation, CD3+ enriched cells were washed three times in RPMI medium containing 1% heat-inactivated FCS, fixed for 1 minute at 0°C in phosphate-buffered saline (PBS) containing 0.05% glutaraldehyde, and neutralized with an equivalent volume of T lymphocyte neutralizing buffer containing 0.2 M glycine. Following a further three washes, the fixed CD3+ cells were resuspended (at a concentration of 2×10^6 cell/ml) in RPMI medium containing 5% heat-inactivated FCS, and stored for up to 7 days at 4°C until used. Prior to use, CD3+ enriched cells were washed twice in complete medium.

FACS staining

Elutriation fractions, RA synovial membrane cells and enriched RA CD3+ve cells were phenotyped using fluorescent conjugated antibodies and analyzed by flow cytometry. In brief, cells (1×10^5/condition) were washed twice in FACS buffer (PBS containing 2% (v/v) FCS and 0.02% sodium azide), pelleted and blocked with 20% human serum. This step and all subsequent incubations were performed for 30 minutes on ice, and the cells washed thrice in FACS buffer after each step. Aliquots were incubated with conjugated anti-CD69 FITC, anti-CD4, anti-CD3 FITC, anti-CDS FITC, anti-CD2 FITC, anti-HLA class 11-FITC, CD45RA-PE, anti-CD45RO-PE (Pharmingen, CA, USA) or CD14-FITC/CD45-PE, CD3-FITC/CD19-PE (Becton Dickinson, CA, USA) (H+L) antibody (PE; Southern Biotechnology Associates, USA) or fluorescein isothiocyanate (FITC; Southern Biotechnology Associates, USA).

Samples were analyzed using the Becton Dickinson Facscan flow cytometer.

T-cell stimulation and fixation

The following challenges were used to stimulate elutriation-enriched T cells:

(i) T_{tcr} cells were selectively stimulated by incubation with immobilised anti-CD3 mAb for 24 hours (OKT3, ATCC, Maryland, USA), which had previously been coated on to a 6-well culture plate at 10 μg/ml (overnight at 4°C) prior to fixation;

(ii) T_{cy} cells were selectively stimulated by incubation with saturating concentrations of a cocktail of TNFα (25 ng/ml), IL-6 (100 ng/ml) and IL-2 (25 ng/ml) for 8 days in culture prior to fixation.

Following stimulation, elutriation-enriched T cells were resuspended at 10^6 cells/ml in culture medium (RPMI 1640) containing 10% heat-inactivated AB+ human serum (BioWhittaker, Verviers, Belgium) and cultured in 6-well cluster culture plates (Falcon) at 37°C in a 5% CO_2/95% air humidified incubator.

Subsequently, normal stimulated T cells and isolated RA CD3+ enriched cells were washed thrice in RPMI medium containing 1% heat-inactivated FCS, fixed for 1 minute at 0°C in PBS containing 0.05% glutaraldehyde,

and neutralized with an equivalent volume of T lymphocyte neutralizing buffer containing 0.2 M glycine.

Monocyte culture

Monocytes were cultured at 1 x 10^6 cells/ml in RPMI 1640 medium (containing 5% heat-inactivated FCS) in round-bottomed 96-well culture plates (Nunc Life Technologies Ltd., Paisley, Scotland) in the presence of fixed T cells at varying T-cell/monocyte ratios ranging from 1:1 to 7:1 (as indicated). In some experiments, a semi-permeable membrane insert (Nunc Life Technologies Ltd., Paisley, Scotland) was fitted into the culture wells to physically separate the monocytes from the T cells. In all experiments, efficient fixation of T cells was tested by incubating an aliquot of T-cells (7 x 10^6 cells/ml) in the absence of monocytes and assaying the amount of TNFα released by the cells under these conditions. Lipopolysaccharide (LPS, 10 ng/ml) was included in experiments as a positive control for monocyte cytokine production.

After 18 hour incubation at 37°C with 5% CO_2, culture supernatants were harvested and stored at -20°C until assayed by ELISA. All experiments were performed at least three times, and the figures show representative examples of these experiments.

Measurement of cytokines by sandwich ELISA

Reagents for the TNFα ELISA were provided by Dr. W. Buurman (Rijks Universiteit Limburg, Maastricht, Netherlands). The ELISA was

performed using immobilized anti-TNFα mAb 61E71 and developed using a rabbit anti-TNFα polyclonal antibody. The rabbit polyclonal antibody was detected using a peroxidase-conjugated goat anti-rabbit IgG (H+L) (Jackson Immunoresearch Laboratories Inc., Westgrove, PE, USA) followed by an appropriate substrate. The sensitivity range of the assay was 1.6 to 5000 pg/ml.

Enzyme inhibitor studies

In all experiments with RA synovial membrane cells, supernatants were harvested at 48 hours since this was found to be optimal for the production of TNFα. Enzyme inhibitors were added to cultures of RA synovial membrane cells for 48 hours at 37°C.

For monocyte/T-cell co-culture experiments, monocytes were cultured with relevant enzyme inhibitors at 37°C for varying periods of time (see legend to Table 2) prior to co-culture with fixed T-cells for 18 hours. Supernatants were harvested and assayed for presence of TNFα by ELISA. Cell suspensions were taken for determination of cell viability.

Analysis of cell viability

Cell viability was assessed using an MTT assay, as described by Denizot and Lang (1986) *J Immunol Methods* **89(2)**, 271-7.

Characterisation of T_{tcr} cell- and T_{cy} cell-induced TNFα production by monocytes

Normal peripheral blood T cells were isolated as described above and activated by stimulation with either (i) immobilized anti-CD3 antibodies (OKT3) for 24 hours, or (ii) a combination of cytokines (IL-2, IL-6 and TNFα) for a period of 8 days, prior to fixation. Both stimulatory challenges induced TNFα production by monocytes in a dose dependent manner, with a co-culture cell ratio of 7:1 (T cells: monocytes) being optimal (data not shown).

Inclusion of a porous membrane insert, which physically separated the two cell populations, significantly reduced TNFα production in monocytes co-cultured with T cells (T cell:monocyte ratio of 5:1) in response to activation with either anti-CD3 antibodies or the combination of cytokines (Figure 1), indicative of the need for cell-cell interactions between T cells and monocytes.

Using the T cell selective stimulatory challenges described above, the T_{tcr} cell- and T_{cy} cell-induced TNFα production by monocytes was characterised as follows:

(i) Over-expression of IκBα inhibits TNFα production in monocytes induced by cytokine stimulated T cells (T_{cy} cells)

TNFα production by monocytes/macrophages in response to certain stimuli is dependent on the activity of the transcription factor NF-κB. For

example, adenoviral gene transfer of the inhibitor of NF-κB (AdvIκBα) has been shown to inhibit lipopolysaccharide- but not zymosan-induced TNFα (Bondeson *et al.*, 1999, *J. Immunol.* **162**, 2939-2945). Hence, the involvement of NF-κB in monocyte TNFα production induced by T_{tcr} and T_{cy} cells was evaluated.

Monocytes were treated with M-CSF for 2 days as described above, infected with adenovirus containing either IκBα (AdvIκBα) or no insert (Adv0). The effect of IκBα over-expression on T cell-induced monocyte (ratio 5:1) TNFα production was then determined.

Data presented in figure 2 demonstrate that over-expression of IκBα significantly ($p<0.001$) reduced TNFα production by 60% from 550 pg/ml (Adv0 controls) to 220pg/ml (AdvIκBα) in M-CSF treated monocytes stimulated by cytokine activated T cells (*i.e.* T_{cy} cells activated by incubation with IL-2, IL-6 and TNFα). In contrast, over-expression of IκBα had little effect on TNFα production in M-CSF monocytes infected with AdvIκBα (1400 pg/ml) compared with untreated Adv0 controls (1500 pg/ml) when stimulated with anti-CD3 antibody activated T cells (*i.e.* T_{tcr} cells).

(ii) PI3 kinase inhibitors attenuate monocyte TNFα production stimulated by T_{tcr} cells but enhance monocyte TNFα production stimulated by T_{cy} cells

The effect of the PI3 kinase inhibitors, wortmannin and LY294002, was investigated on T cell-induced TNFα production by monocyte.

Wortmannin and LY294002 significantly *inhibited* monocyte TNFα production induced by anti-CD3 antibody stimulated T-cells (*i.e.* T_{tcr} cells) in a dose dependent manner (figure 3a and 3c). The IC_{50} values for wortmannin (0.05 nM) and LY294002 (0.07 μM) were indicative of this being a PI3 kinase-mediated event.

In contrast, wortmannin and LY294002 significantly *enhanced* monocyte TNFα production (by about 3-4 fold) induced by cytokine stimulated T cells (*i.e.* T_{cy} cells) (figure 3b and 3d) in a similar manner to that previously observed with LPS.

Kinase p70 S6 is a downstream effector of PI3 kinase (Monfar *et al.*, 1995, *Mol. Cell Biol.* **15**:326-337), the activity of which can be blocked with the drug rapamycin. Rapamycin was found to have no effect on monocyte TNFα production induced by either cytokine stimulated (IL-2, IL-6 and TNFα) or anti-CD3 antibody stimulated T cells (results not shown).

In addition to inhibiting PI3 kinase activity, the fungal product wortmannin has also been shown to inhibit other signalling pathways, including phospholipase A_2 (PLA_2) (Cross *et al.*, 1995). To test whether the effect of wortmannin on T_{cy} cell- and T_{tcr} cell-induced TNFα production by monocytes involved the PLA_2 signalling pathway, the effect of a second PLA_2 inhibitor, arachidonyl trifluoromethyl ketone analogue (AKTA), was tested.

Unlike LPS-induced stimulation of monocytes, AKTA had no effect on TNFα production following stimulation with T-cells cultured with either anti-CD3 antibodies or cytokines (IL-2, IL-6 and TNFα) (results not shown). These observations indicate that the effect of wortmannin on T_{cy} cell- and T_{tcr} cell-induced TNFα production by monocytes is not mediated by PLA_2.

TNFα production in synovial fluid from rheumatoid arthritis (RA) patients: identification of the T cell subtypes involved

Experiments were performed to investigate whether T cells enriched from RA synovial tissue could induce TNFα synthesis in normal resting monocytes without further activation (*i.e.* without incubation with anti-CD3 antibodies or a cocktail of cytokines). T cells were enriched from RA synovial mononuclear cells (MNC) using anti-CD3 'detach a bead' at 4°C as described above. In replicate experiments (n=3), RA CD3+ve T cells were to be predominantly CD4+ CD45RO+, although CD8+ and CD45RA cells were also present. The T cell activation markers, HLA-DR and CD69 were also present, suggesting that RA CD3+ cells were of the "memory" phenotype and activated (see Table 1).

Table 1

Surface Marker	% cells stained
CD4	57.73
CD8	30.50
CD69	37.53
CD45RA	49.77
CD45RO	95.34
HLA-DR	52.48
CD14	3.72

Table 1 shows the cellular phenotype of RA CD3-positive cells.

RA CD3-positive cells were isolated from RA synovial mononuclear cells by direct, positive selection on anti-CD3 antibody coated Dynabeads. FACS analysis was performed using a Becton Dickinson Facscan flow cytometer. Data are presented as percentage of stained cells compared with isolated matched controls, and are representative of two experiments using different donors.

Monocytes isolated by elutriation were cultured with fixed RA CD3+ enriched cells for 18 hours. Data presented in Figure 4 demonstrate that RA CD3+ cells induced TNFα production in normal monocytes in a dose dependent manner with a T cell:monocyte ratio of 5:1 being the most effective (inducing 300 pg/ml TNFα). Fixed RA CD3+ enriched cells cultured alone did not release TNFα above the lowest limit of detection in the ELISA (50pg/ml). Furthermore, monocyte derived TNFα induced by fixed RA CD3+ enriched cells was inhibited by about 90% when the two

cell populations were physically separated, preventing any contact-mediated events from occurring.

In addition, TNFα levels were significantly reduced in RA synovial cultures in which the CD3+ve T cells had been depleted. Specifically, TNFα levels in 2-day cultures were reduced by 71% from 512 pg/ml in the total synovial cell cultures to 148 pg/ml in the cell-depleted culture (data not shown). At 5 days in culture, the total synovial cell population produced 151 g/ml TNFα with less than 20 pg/ml produced in the T cell-depleted cultures.

Following the characterisation of T_{tcr} cell- and T_{cy} cell-induced TNFα production by monocytes described above, further experiments were performed to identify which subtype(s) of T cell were involved in TNFα production in RA synovial fluid.

(i) Effect of over-expression of IκBα on monocyte TNFα production induced by T cells derived from RA synovial tissue.

It had previously been demonstrated that the spontaneous production of TNFα in RA synovial tissue cultures was inhibited (by more than 80%) following blockade of the transcription factor NFκB, using an adenovirus over-expressing the inhibitor IκBα (Foxwell *et al.*, 1998). As blockade of NFκB with an adenovirus expressing IκBα also discriminated between T_{cy} and T_{tcr} cell-induced monocyte TNFα production, comparable experiments were performed to determine whether NFκB played an

important regulatory role in the production of TNFα in normal peripheral blood monocytes stimulated with fixed RA T cells.

Peripheral blood monocytes were treated with M-CSF for 2 days to enable infection with either empty adenovirus (Adv0) or with adenovirus expressing IκBα (multiplicity of infection, or m.o.i., from 20 to 80:1). RA T cells were enriched from RA synovial tissue as described above, fixed and co-cultured with adenovirus-infected monocytes at a T cell: monocyte ratio of 3:1 for 18 hours.

Monocyte TNFα production induced by RA CD3+ enriched cells was inhibited by more than 70% ($p<0.0001$) when the monocytes were infected with adenovirus over-expressing IκBα (m.o.i 80:1) (Figure 5). TNFα levels in IκBα-infected monocytes stimulated with RA T cells were 83 ± 11 pg/ml compared with 300 ± 12 pg/ml in Adv0-infected monocytes ($p<0.0001$). At a m.o.i. of 40:1, TNFα production was also inhibited ($>50\%$) but, due to insufficient RA T cells, this point was not performed in triplicate and hence statistical analysis could not be performed. No effect was observed with a m.o.i of 20:1. Thus, monocyte TNFα production induced by RA CD3+ enriched cells was similar to that induced by T_{cy} cells and spontaneous TNFαproduction in RA synovial joint cells but was unlike that induced by anti-CD3 antibody stimulated T_{tcr} cells.

As a positive control, monocytes from the same elutriation were also infected with adenovirus overexpressing IκBα and stimulated with LPS. As reported previously (Foxwell *et al.*, 1998), over-expression of IκBα at

a m.o.i. of 80:1 inhibited LPS-induced TNFα from 3497±805 pg/ml to 1025±235p,,/ml (data not shown).

(ii) Effect of PI3 kinase inhibitors on TNFα production in RA synovial cultures and RA T cell-induced monocytes

Since PI3 kinase inhibitors were found to discriminate between T_{cy} and T_{tcr} induced monocyte TNFα production, experiments were performed to determine whether PI3 kinase played an important regulatory role in the spontaneous production of TNFα from RA synovial joint cell cultures. The effect of the PI3 kinase inhibitors, wortmannin (Figure 6a) and LY294002 (Figure 6b) on spontaneous cytokine production from eight individual RA synovial membrane cultures was investigated.

Both wortmannin and LY294002 significantly enhanced spontaneous TNFα production (by about 9- and 2-fold, respectively) in RA mononuclear cell cultures in a dose dependent manner (figure 6a and 6b).

The effect of wortmannin and LY294002 upon monocyte TNFα production induced by RA T cells was also investigated (see table 2). Normal monocytes isolated by elutriation were treated with selective enzyme inhibitors for the designated times prior to co-culture with fixed rheumatoid CD3+ T cells at a ratio 5:1 (T cells to monocytes) for 18 hours. Due to the limited number of RA CD3+ enriched cells, only one concentration of each inhibitor was used, *i.e.* the concentration shown to maximally inhibit monocyte TNFα production induced by fixed activated T cells. Unlike anti-CD3 antibody stimulated T cells, but similar to

cytokine stimulated T cells and the spontaneous production of TNFα in RA synovial joint cells, TNFα production induced by RA CD3+ enriched cells was enhanced by wortmannin and LY294002. Thus, TNFα levels were increased from 686±59 pg/ml to 7333±304 pg/ml with wortmannin (500 nM), and from 686±59 pg/ml to 883±304 pg/ml with LY294002 (50 μM).

Experiments were also performed to determine whether the PLA_2, AKTA, displayed similar effects on TNFα synthesis to that of wortmannin. Consistent with T_{cy} cell-induced stimulation of monocytes, AKTA had no effect on TNFα production in monocytes stimulated by RA T-cells (Table 2). Furthermore, the inclusion of rapamycin (10 μM) had no significant effect on TNFα synthesis.

In summary, these findings indicate that T_{cy} cells are involved in the induction of TNFα production by monocytes in RA synovial tissue. Hence, T_{cy} cells represent a novel therapeutic target for the development of treatments of chronic inflammatory disorders.

Table 2

Inhibitor	TNFα (pg/ml)	Percent change
Untreated (control)	686 ± 59	-
LY294002 (50 μM)	883 ± 38	+29%
Wortmannin (500 nM)	7333 ± 304	+968%
AKTA (5 mM)	606 ± 16	-11%
Rapamycin (10 μM)	399 ± 21	-42%

Table 2 shows the effect of incubation with LY294004, wortmannin, AKTA and rapamycin on monocyte TNFα production induced by rheumatoid arthritis T cells.

CD3+ enriched cells were isolated from RA synovial mononuclear cells by direct, positive selection on anti-CD3 antibody coated Dynabeads. Following fixation, RA T cells were incubated for in the absence ('untreated') or presence of LY294002 (50 μM, 30 minutes), Wortmannin (500 nM, 30 minutes), AKTA (5 mM, 30 minutes) or Rapamycin (10 μM, 60 minutes). RA T cells were then co-cultured with monocytes for 18 hours at a ratio 5:1 (T cells to monocytes). Culture supernatants were assayed for TNFα content. Data are expressed as means ± SD, and are representative of three experiments performed using different donors.

Methods of screening for compounds that selectively target T_{cy} cells

(1) Assay for NFκB inhibition

Monocyte purification: Human peripheral blood monocytes are isolated from single donor plateletpheresis residues by Ficol/Hypaque centrifugation, as described above. Monocyte purity is then assessed by flow cytometry using fluorochrome-conjugated anti-CD45 and anti-CD14 monoclonal antibodies (Becton Dickinson, Oxford, UK), which routinely reveals that greater than 85% of cells express CD3 or CD14, respectively.

Monocyte culture: Upon isolation, monocytes are cultured in complete medium at 4×10^6 cells/ml in 96-well culture plates (Nunc Life Technologies Ltd, Paisley, Scotland). At the start of the culture period, cells are divided into the following treatment groups:

(i) One control group of cells is incubated in the absence of any drug challenge (negative control group);
(ii) At least one group of cells is incubated with a test compound (a test group); and
(iii) A final group of cells is incubated with 10 μg/ml LPS (positive control group).

It will be appreciated that the concentration of the test compound in the culture medium of the test group and the duration of this initial incubation period may be varied. Typically, a range of drug concentrations and

incubation duration periods will be used when testing a given compound to be tested.

Following this initial stimulatory incubation period, the monocytes are cultured for a further 18 hours at 37°C with 5% CO_2 in air.

Electrophoretic mobility shift assay (EMSA): Nuclear extracts (10 μg) are prepared as described in Dent and Latchman (1993) *Transcription factors: A practical approach*, Oxford University Press. The extracts were then mixed with 5x binding buffer (100 mM Tris pH8.0, 20 mM KCl, 10 mM $MgCl_2$, 60% glycerol, 6 mM DTT), 1 μl poly(dI).poly(dC) (2.5 mg/ml) and the volume was made up to 20 μl with distilled water. After equilibration of the mixture for 5 minutes at room temperature, 5 x 10^4 cpm of the following double-stranded oligonucleotide probe was added (with or without unlabelled competitor DNA):

5'- [α-^{32}P]dCTP-GAT **CGG GAC TTT** CCC-3'
3'-**TCC CTG AAA GGG** TAC- [α-^{32}P]dCTP-5'

The mixture containing the labelled probe was then left at room temperature for 20 minutes (the NF-κB promoter sequence in shown above in bold font).

Alternatively, commercially available transcription factor consensus sequence oligonucleotides may be radiolabelled and used, such as oligonucleotide E3241 (Promega). It will be appreciated that such oligonucleotides may be radiolabelled by any methods known in the art, for example by enzyme-catalysed addition of ^{32}P-labelled dATP or dCTP.

Samples were run on a pre-electrophoresed native (0.09M Tris borate, 2 mM EDTA, pH8.0, TBE) 5% polyacrylamide gel for 90 minutes at 200 V. The gel was dried and autoradiography was performed by exposure to Hyperfilm MP (Amersham, UK). Autoradiograms were analysed by densitometry (Biorad GS670, Biorad, Watford, UK).

NF-κB inhibition is deemed to exist if the binding of NF-κB to its promoter on DNA oligonucleotides (as measured by densitometric analysis of the autoradiogram) is reduced to no more than 50% compared to the binding of nuclear extracts prepared from monocytes that have not been pre-incubated with the test compound. Preferably, the binding is no more than 20%, 10%, 5% or 1%. Ideally, the binding is substantially zero.

As a positive control in the EMSA assay (*i.e.* to inhibit NF-κB), monocytes may be infected with adenovirus containing IκBα (AdvIκBα) at an m.o.i. of 100:1.

Reporter gene assay: NF-κB gene expression may be measured by a reporter gene assay using a cell line stably transfected with the NF-κB gene coupled to a β-galactosidase gene, for example as described in Matilla *et al.* (1990) *EMBO J.* **9(13)**:4425-4433.

Cells are seeded at 2 x 10^6/ml in 100 μl culture medium (RPMI 1640) containing 10% heat-inactivated foetal calf serum (BioWhittaker, Verviers, Belgium) and cultured overnight (at 37°C in a 5% CO_2/95% air

humidified incubator) with and without a compound to be tested. Following incubation, the cells are lysed by the addition of 20μl 1% Triton X-100. β-galactosidase activity was assayed by adding 25 μl of 8 mg/ml chlorophenol red β-D-galactopyranoside (Boehringer Mannheim, Germany) and incubating the samples at 37°C for 2 to 4 hours. Absorbance was measured at 574 nm on an automatic plate reader (for example, Labsystems Multiscan Bichromatic) and data were analysed with Deltasoft software (Biometallics).

NF-κB inhibition is deemed to exist if absorbance at 574 nm is reduced to no more than 50% compared to absorbance in lysates from cells which have not been incubated with the test compound, Preferably, the absorbance at 574 nm is no more than 20%, 10%, 5% or 1%. Ideally, the absorbance at 574 nm is substantially zero.

(2) Assay for PI3 kinase activation

PI3 kinase activity may be assayed by any method known in the art, for example using the assay described in Crawley *et al* (1996) *J Biol Chem* **271(27)**, 16357-16362, and Fukni and Hanafusa (1994) *Mol cell Biol* **9**, 1651-1658.

Preferably, PI3 kinase activity is assayed as follows:

Monocyte purification: Human peripheral blood monocytes are isolated from single donor plateletpheresis residues by Ficol/Hypaque centrifugation, as described above. Monocyte purity is then assessed by

flow cytometry using fluorochrome-conjugated anti-CD45 and anti-CD14 monoclonal antibodies (Becton Dickinson, Oxford, UK), which routinely reveals that greater than 85% of cells express CD3 or CD14, respectively.

Monocyte culture: Upon isolation, monocytes are cultured in complete medium at 4×10^6 cells/ml in 96-well culture plates (Nunc Life Technologies Ltd, Paisley, Scotland). At the start of the culture period, cells are divided into the following treatment groups:

(i) One control group of cells is incubated in the absence of any drug challenge (negative control group);

(ii) At least one group of cells is incubated with a test compound (a test group); and

(iii) A final group of cells is incubated with IL-10 (100 mg/ml) for 2 minutes, as a positive control.

It will be appreciated that the concentration of the test compound in the culture medium of the test group and the duration of this initial incubation period may be varied. Typically, a range of drug concentrations and incubation duration periods will be used when testing a given compound to be tested.

Following this initial stimulatory incubation period, the PI3 kinase inhibitors wortmannin or LY294002 (Sigma, Poole, UK) are added to the monocyte cultures for 15 minutes to block PI3 kinase activation. After 18 hours in culture at 37°C with 5% CO_2 in air, supernatant aliquots are

harvested (200 μl/well, 3 wells/treatment group) and stored at 20°C until used.

Immunoprecipitation and in vitro kinase assays: Following stimulation, monocytes are lysed at a density of 5 x 10^6 cells/ml in PI3 kinase lysis buffer (20 mM Tris-HCl pH7.5, 150 mM NaCl, 5 mM EDTA, 1% Nonidet-P40), supplemented with 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotonin, 1 μg/ml pepstatin and 10 μg/ml leukopeptin (Calbiochem). To the supernatants are added monoclonal antibodies (U5 mAbs) directed against the p85α subunit of PI3 kinase (available from Dr Cantrell, ICRF, London, UK). After 30 minutes on ice, 20 μl of the protein G-Sepharose was added and the lysates rotated at 4°C for 2 hours.

PI3 kinase assay: Beads containing immunoprecipitates are washed three times for 5 minutes each wash in PI3 kinase lysis buffer, once in PBS, twice in 500 mM lithium chloride, once in water and once in PI3 kinase assay buffer (40 mM Tris-HCl pH7.5, 200 mM NaCl, 1 mM EGTA). Immunoprecipitates are then resuspended in 40 μl of PI3 kinase assay buffer. Upon resuspension, 50 μl of lipid substrate mixture is added, which contains 1 mg phosphatidyl-inositol(4,5)P_2 (PtdIns-4,5-P_2) and 1 mg phosphatidylserine (PtdS) (both from Sigma, UK) made up in 2 ml of 25 mM HEPES/1 mM EDTA, and dispersed by sonication in three 15-second bursts at 4°C). The reaction is initiated by addition of 5 μCi [γ-32P]-ATP and 100 mM ATP. The samples are incubated at room temperature for 15 minutes and the reaction quenched using 100 μl of 1 M HCl and 200 μl of 1:1 chloroform:methanol. The resultant lipid layer is

then removed and dried *in vacuo*. The dried samples are resuspended in 50 μl chloroform, applied to a 1% oxalate-sprayed thin layer chromatography (TLC) plate and developed in propan-1-ol:2 M glacial acetic acid (65:35 v/v). Reaction products (*i.e.* phosphatidylinositol-3,4,5-triphosphate, PtdIns-3,4,5-P_3) are visualised by autoradiography using Hyperfilm MP (Amersham, UK).

PI3 kinase is deemed to have been activated if there is an increase in PI3 kinase activity (as measured by densitometric analysis of the signal corresponding to the PI3 kinase reaction product on the autoradiogram) equivalent to at least 50% of the increase induced in the IL-10 stimulated positive control group. Preferably, the increase in activity is equivalent to at least 70%, 80% or 90% of that in the positive control group. Ideally, the increase in activity is greater than that in the positive control group.

Therapeutic use of compounds in the treatment of rheumatoid arthritis

An amount of a compound which is an inhibitor of NFκB is dissolved in sterile, non-pyrogenic water or isotonic saline. The solution is then injected intra-articularly (using a hypodermic needle) into the knee joint of a patient suffering from rheumatoid arthritis of the knee joint. Preferably, the solution is administered at regular intervals (*e.g.* daily, twice weekly, weekly or monthly) for a prolonged period, such that the symptoms associated with rheumatoid arthritis (*e.g.* inflammation of the synovium of the knee and/or joint immobility) are eased or are prevented from worsening.

It will be appreciated that the NFκB inhibitor solution may be used immediately upon preparation or may be stored in sterile containers (*e.g.* glass ampoules) prior to use. If stored for a prolonged period, suitable preservative agents may be added to the solution.

CLAIMS

1. A method of treatment of a chronic inflammatory disease in a patient, the method comprising the administration to the patient of a compound that selectively inhibits T_{cy} cells.

2. A method according to claim 1 wherein said compound selectively inhibits T_{cy} cell-induced release of one or more pro-inflammatory cytokines from monocytes.

3. A method according to claim 2 wherein the cytokine is tumour necrosis factor-α.

4. A method according to any one of claims 1 to 3 wherein said compound selectively inhibits NF-κB.

5. A method according to any one of claims 1 to 3 wherein said compound selectively activates PI3 kinase.

6. A method of identifying a compound with efficacy in the treatment of a chronic inflammatory disease comprising the step of testing the compound for an ability to selectively inhibit T_{cy} cells.

7. A method of identifying a compound with efficacy in the treatment of a chronic inflammatory disease comprising the step of testing the compound for an ability to selectively inhibit T_{cy} cell-induced release of one or more pro-inflammatory cytokines from monocytes.

8. A method according to claim 7 wherein the cytokine is tumour necrosis factor-α.

9. A method according to claim 8 wherein said method comprises the following steps:

(i) pre-incubating separate cultures of T_{cy} cells and T_{tcr} cells with a compound to be tested;
(ii) resuspending said T_{cy} cells and T_{tcr} cells in the absence of the test compound;
(iii) stimulating monocytes by co-culturing with said resuspended T_{cy} cells and T_{tcr} cells; and
(iv) assaying for TNFα production by said stimulated monocytes.

10. A method according to any one of claims 6 to 9 wherein the chronic inflammatory disease is a disease of humans.

11. A method according to any one of claims 6 to 10 wherein the chronic inflammatory disease is rheumatoid arthritis.

12. A method according to any one of claims 6 to 11 wherein testing the compound for an ability to selectively inhibit T_{cy} cells or selectively inhibit T_{cy} cell-induced release of one or more pro-inflammatory cytokines from monocytes comprises determining whether the compound exhibits NF-κB inhibition.

13. A method according to claim 12 wherein NF-κB inhibition is constituted by a reduction in the binding of nuclear extracts, derived from monocytes exposed to the compound, to an NFκB promoter DNA oligonucleotide.

14. A method according to claim 13 wherein a reduction in the binding of nuclear extracts, derived from monocytes exposed to the compound, to an NFκB promoter DNA oligonucleotide is determined by an electrophoretic mobility shift assay (EMSA).

15. A method according to any one of claims 12 to 14 wherein NF-κB inhibition is deemed to exist if the binding of NF-κB to an NFκB promoter DNA oligonucleotide is reduced to no more than 50%, preferably no more than 20%, 10%, 5% or 1%, and most preferably is substantially zero.

16. A method according to claim 12 wherein NF-κB inhibition is constituted by a reduction in expression of the NF-κB gene.

17. A method according to claim 16 wherein a reduction in the expression of the NF-κB gene is determined by a reporter gene assay.

18. A method according to claim 17 wherein the reporter gene assay comprises coupling a β-galactosidase gene to the NF-κB gene and determining a reduction in β-galactosidase activity.

19. A method according to claim 18 wherein β-galactosidase activity is reduced to no more than 50%, preferably no more than 20%, 10%, 5% or 1%, and most preferably is substantially zero.

20. A method according to any one of claims 6 to 11 wherein testing the compound for an ability to selectively target T_{cy} cells or selectively inhibit T_{cy} cell-induced release of one or more pro-inflammatory cytokines from monocytes comprises determining whether the compound exhibits PI3 kinase activation.

21. A method according to claim 20 wherein PI3 kinase activation is constituted by an increase in PI3 kinase activity in monocytes exposed by the compound.

22. A method according to claim 21 wherein PI3 kinase activation is deemed to exist if there is an increase in PI3 kinase activity equivalent to at least 50% of the increase induced by IL-10 stimulation (100 ng/ml for 2 minutes), preferably at least 70%, 80% or 90%, and most preferably greater than the increase induced by IL-10 stimulation.

23. A compound identifiable or identified as having efficacy in the treatment of a chronic inflammatory disease by a method according to any one of claims 1 to 9.

24. A compound according to claim 23 for use in medicine.

25. Use of a compound according to claim 23 in the preparation of a medicament for the treatment of a chronic inflammatory disease.

26. The use according to claim 25 wherein the chronic inflammatory disease is rheumatoid arthritis.

27. A pharmaceutical formulation comprising a compound according to claim 23 and a pharmaceutically acceptable carrier.

ABSTRACT

The invention provides a method of treatment of a chronic inflammatory disease (such as rheumatoid arthritis) in a patient, the method comprising the administration to the patient of a compound that selectively inhibits T_{cy} cells. Preferably, said compound selectively inhibits T_{cy} cell-induced release of one or more pro-inflammatory cytokines from monocytes. Advantageously, said compound inhibits NF-κB. Conveniently, said compound activates PI3 kinase. The invention further provides a method of identifying a compound with efficacy in the treatment of a chronic inflammatory disease comprising the step of testing said compound for an ability to selectively inhibit T_{cy} cells. Preferably, said method of identifying a compound with efficacy in the treatment of a chronic inflammatory disease comprises the step of testing said compound for an ability to selectively inhibit T_{cy} cell-induced release of one or more pro-inflammatory cytokines from monocytes. Conveniently, the pro-inflammatory cytokine is tumour necrosis factor α (TNFα). The invention further provides compounds identifiable or identified by said methods and the use of said compounds in medicine.

FIG. 1

Figure 1

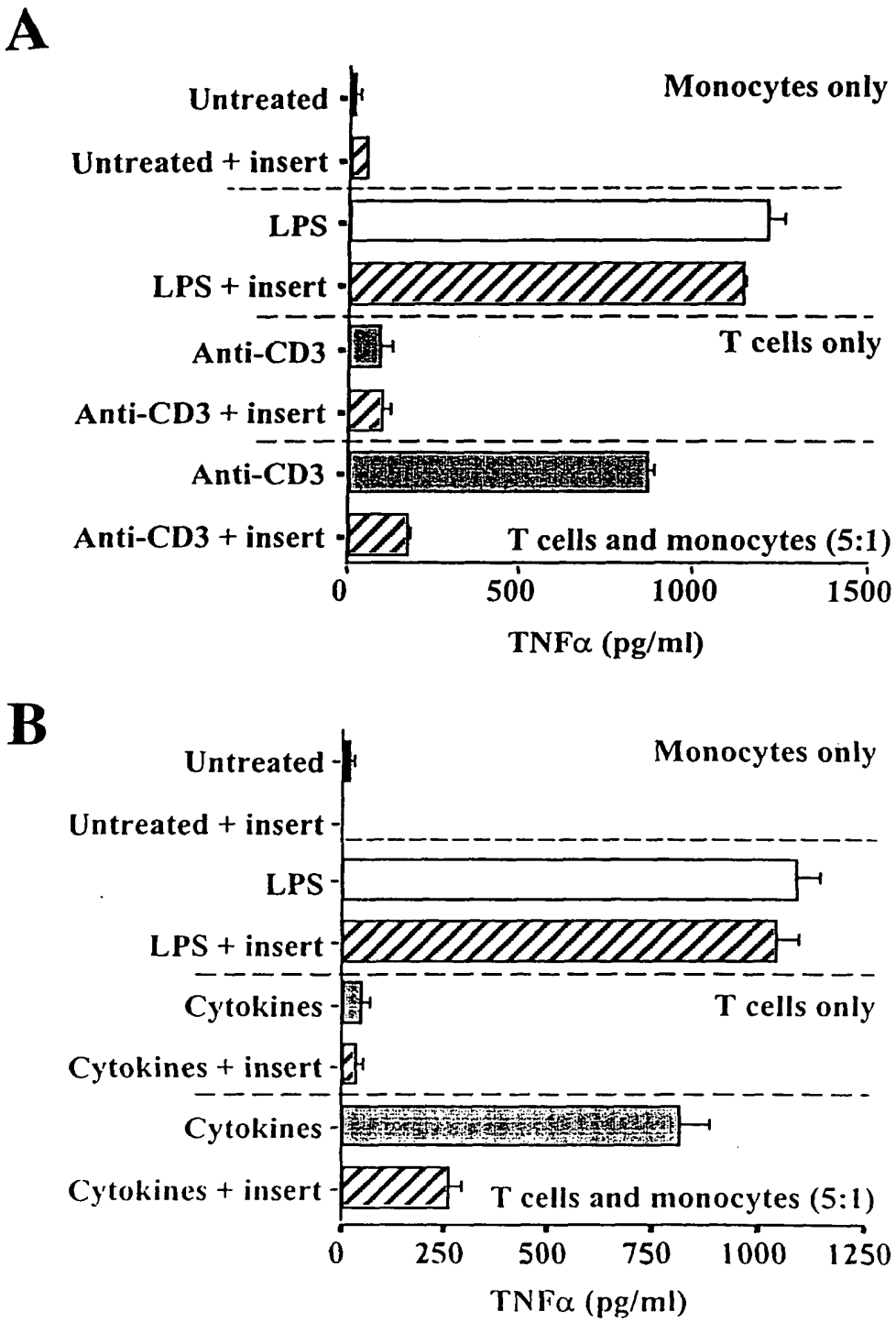

A
Untreated
Untreated + insert
LPS
LPS + insert
Anti-CD3
Anti-CD3 + insert
Anti-CD3
Anti-CD3 + insert
Monocytes only
T cells only
T cells and monocytes (5:1)
0
500
1000
1500
TNFα (pg/ml)
B
Untreated
Untreated + insert
LPS
LPS + insert
Cytokines
Cytokines + insert
Cytokines
Cytokines + insert
Monocytes only
T cells only
T cells and monocytes (5:1)
0
250
500
750
1000
1250
TNFα (pg/ml)

Figure 2

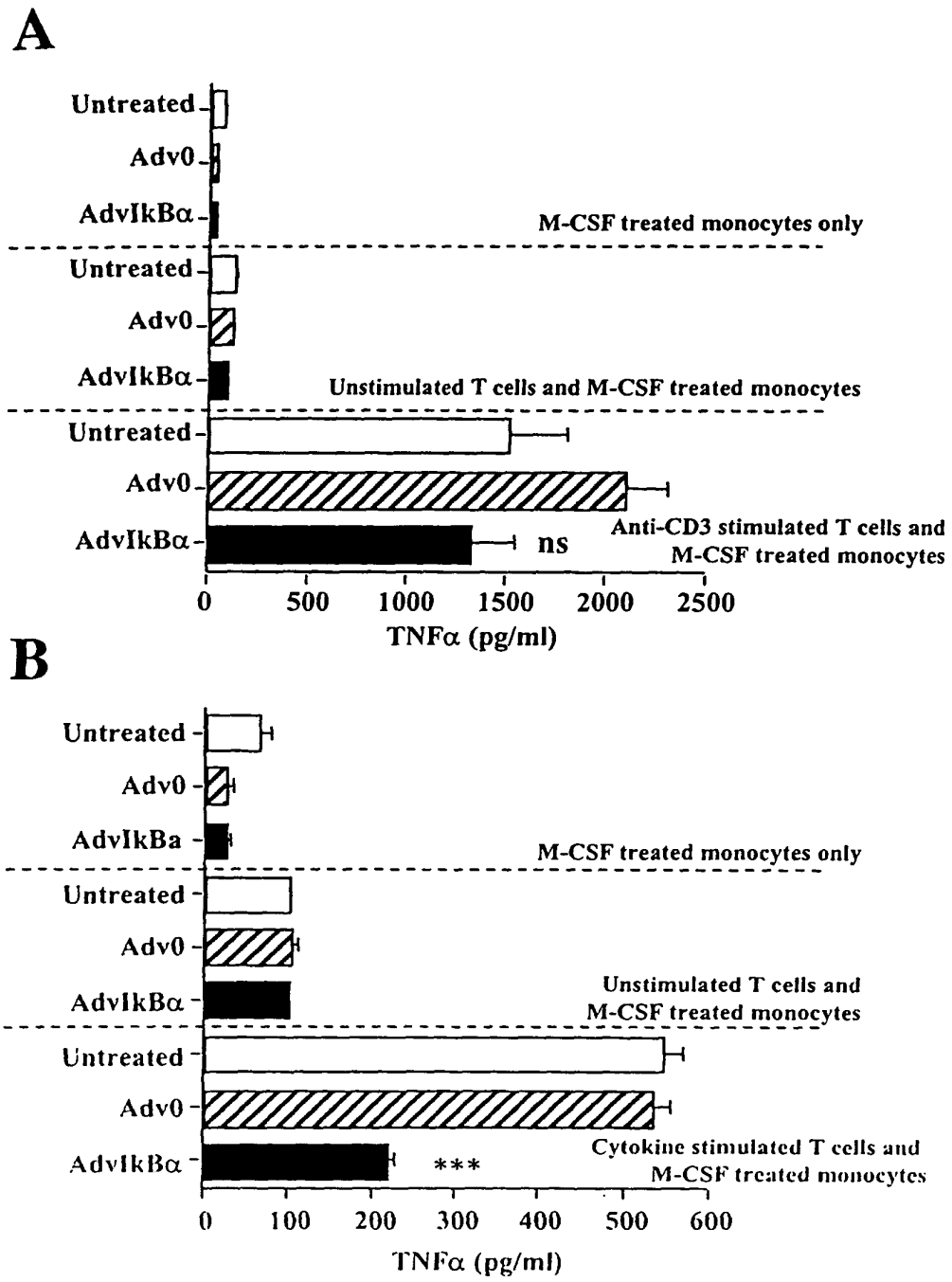

A
Untreated
Adv0
AdvIkBα
M-CSF treated monocytes only
Untreated
Adv0
AdvIkBα
Unstimulated T cells and M-CSF treated monocytes
Untreated
Adv0
AdvIkBα
ns
Anti-CD3 stimulated T cells and M-CSF treated monocytes
0
500
1000
1500
2000
2500
TNFα (pg/ml)
B
Untreated
Adv0
AdvIkBa
M-CSF treated monocytes only
Untreated
Adv0
AdvIkBα
Unstimulated T cells and M-CSF treated monocytes
Untreated
Adv0
AdvIkBα
***
Cytokine stimulated T cells and M-CSF treated monocytes
0
100
200
300
400
500
600
TNFα (pg/ml)

Figure 3

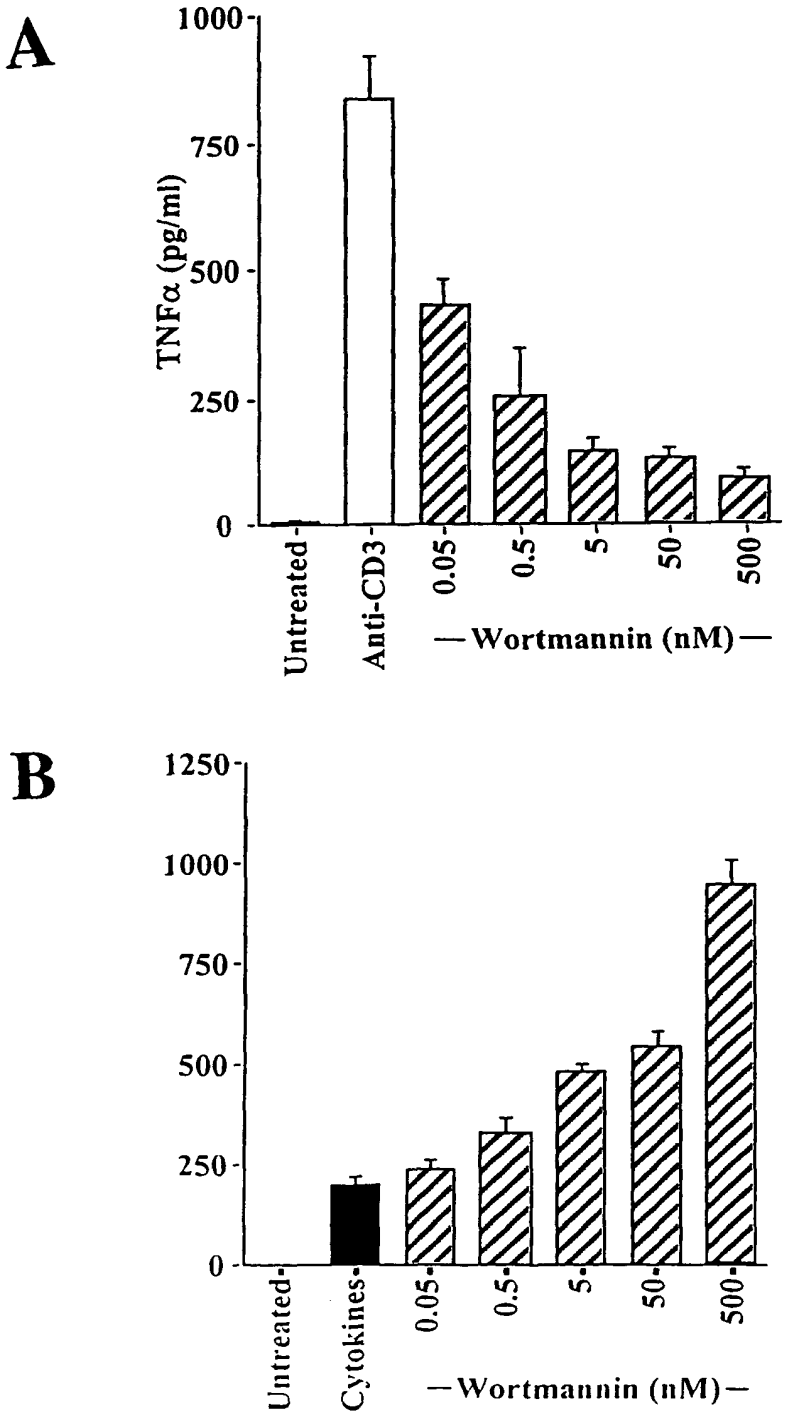

A
1000
750
500
250
0
TNFα (pg/ml)
Untreated
Anti-CD3
0.05
0.5
5
50
500
—Wortmannin (nM)—
B
1250
1000
750
500
250
0
Untreated
Cytokines
0.05
0.5
5
50
500
—Wortmannin (nM)—

Figure 3 (continued)

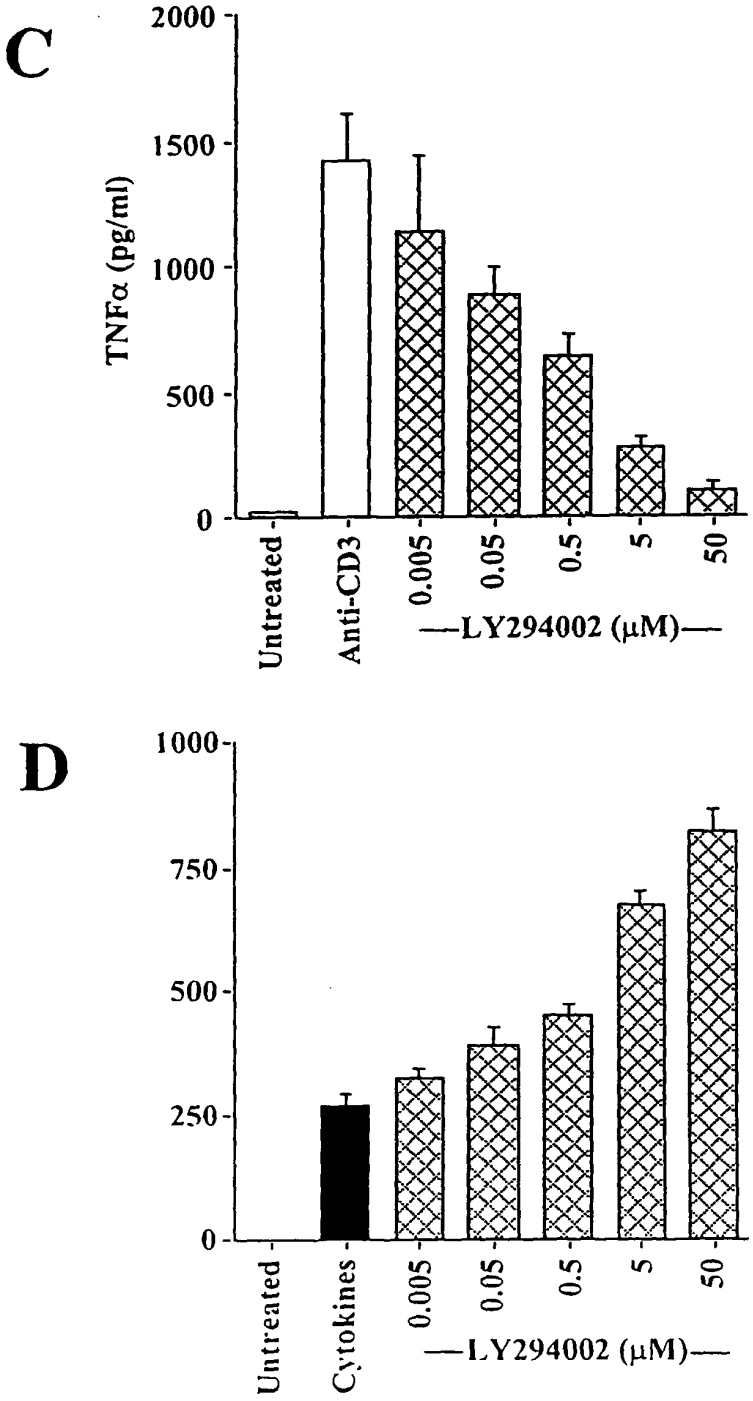


Figure 4

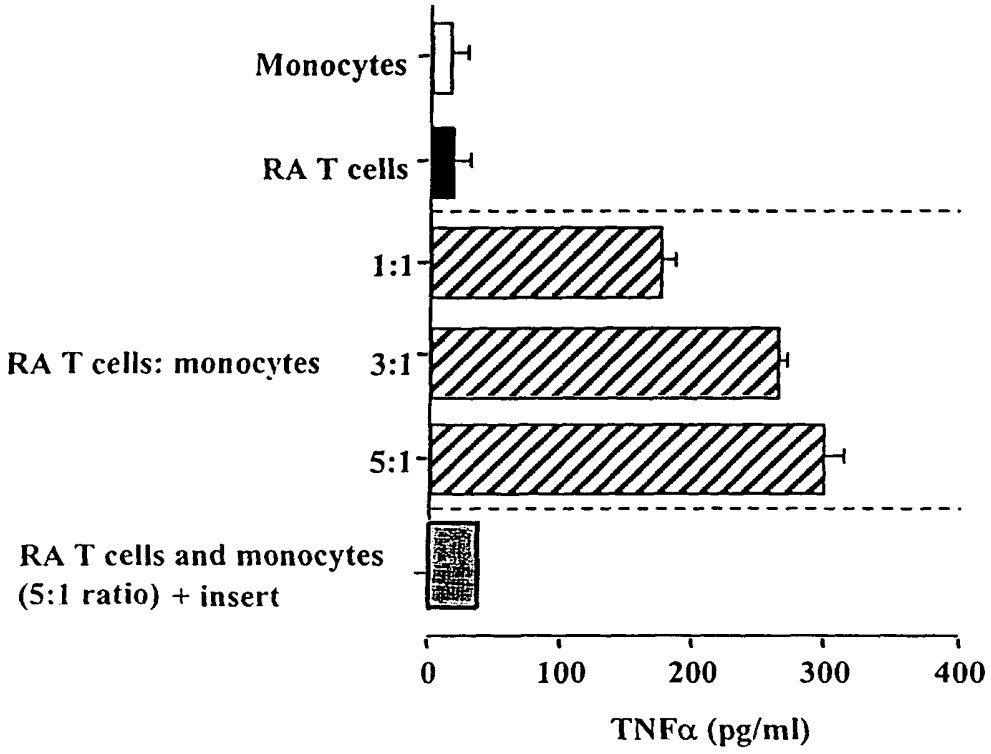

Monocytes
RA T cells
1:1
RA T cells: monocytes
3:1
5:1
RA T cells and monocytes
(5:1 ratio) + insert
0
100
200
300
400
TNFα (pg/ml)

Figure 5

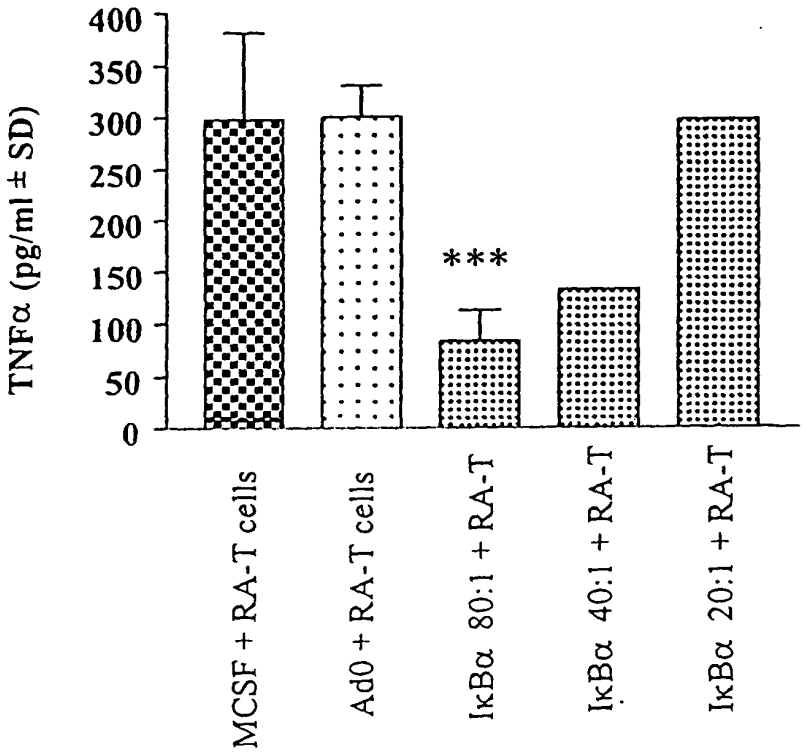
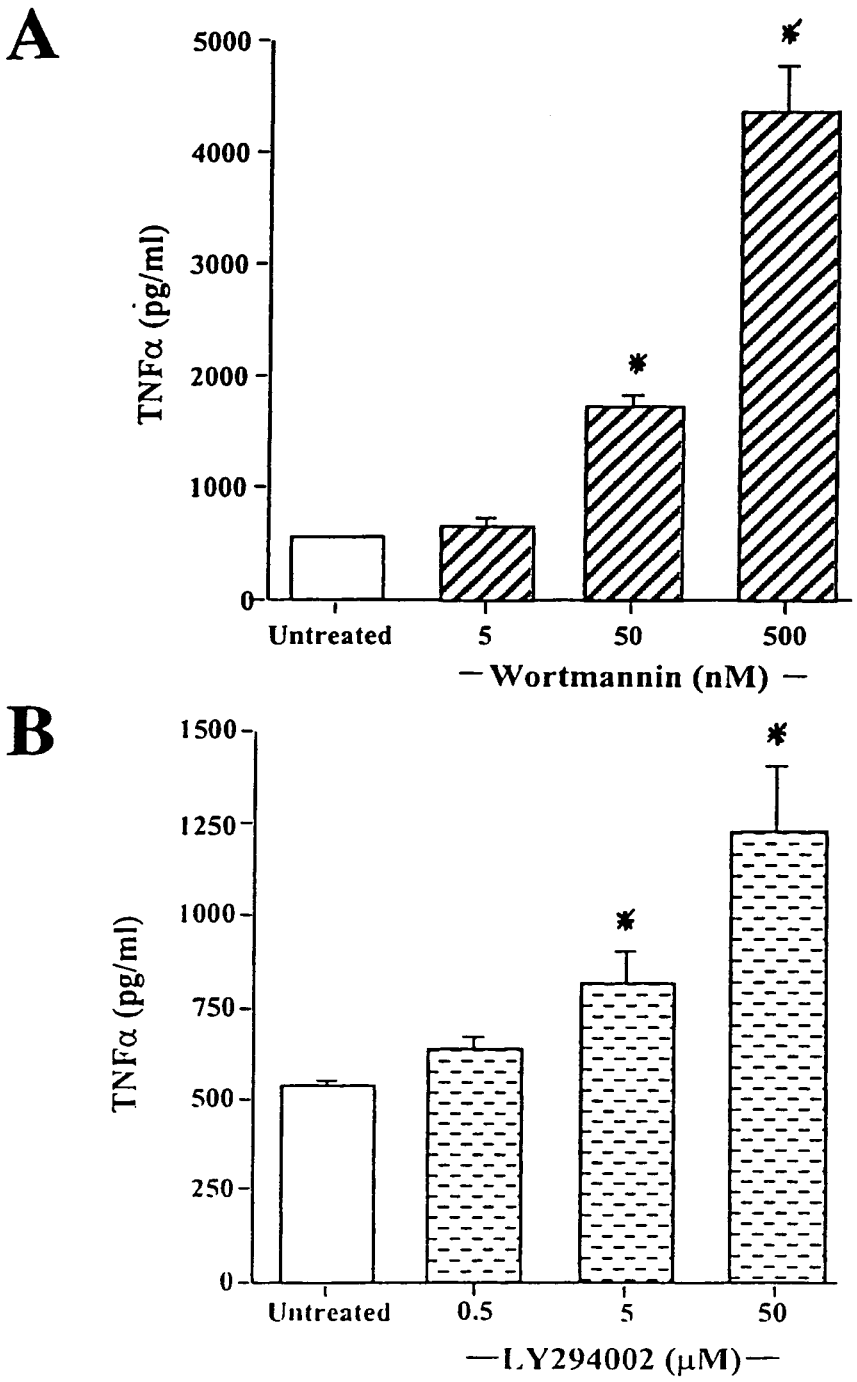

TNFα (pg/ml ± SD)
400
350
300
250
200
150
100
50
0
***
MCSF + RA-T cells
Ad0 + RA-T cells
IκBα 80:1 + RA-T
IκBα 40:1 + RA-T
IκBα 20:1 + RA-T

Figure 6


A
5000
4000
3000
2000
1000
0
TNFα (pg/ml)
*
*
Untreated
5
50
500
—Wortmannin (nM) —
B
1500
1250
1000
750
500
250
0
TNFα (pg/ml)
*
*
Untreated
0.5
5
50
—LY294002 (μM)—

PCT NO : GB00 / 03660

Fraser Davis